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Production of Hydroxy Acids through Selective Double Oxidation of Diols by a Flavoprotein Alcohol Oxidase

Caterina Martin[†], Milos Trajkovic[†] and Marco W. Fraaije^{*}

Abstract: Flavoprotein oxidases can catalyse oxidations of alcohols and amines by using merely using molecular oxygen as oxidant, making this class of enzymes appealing for biocatalysis. The FAD-containing alcohol oxidase from *P. chrysosporium* was found to be able to perform double and triple oxidations on a range of aliphatic diols. Interestingly, depending on the diol substrate, these reactions result in formation of lactones or hydroxy acids. For example, diethylene glycol could be selectively and fully converted into 2-(2-hydroxyethoxy)acetic acid. Such a facile cofactor-independent biocatalytic route towards hydroxy acids opens up new avenues for the preparation of polyester building blocks.

Oxygen-containing heterocycles (O-heterocycles) form a class of compounds proven to be relevant in the polymers, fuel and in the medical fields.^[1–4] Some of these O-heterocycles such as lactones and in particular 1,4-dioxan-2-one can be used for synthesis of biodegradable polyesters that find countless clinical applications thanks to their biocompatibility and strength properties.^[5,6] Bioabsorbable polymers derived from 1,4-oxathian-2-one possess similar characteristics to polydioxanone, but they are not as studied and commercially used, probably due to the poor yield of synthesis of 1,4-oxathian-2-one.^[7] The chemical routes to synthesize some of these compounds often require expensive transition metal catalysts (Au or Pd catalysts) that increase the production costs.^[8] Moreover biodegradable polymers for biomedical applications need to be metal free.^[9] Enzymatic synthesis of lactones as polyesters building blocks gained great interest as environmentally sustainable alternative to current chemical methods. The most common biocatalysts to produce lactones are Baeyer-Villiger monooxygenases (BVMOs) and alcohol dehydrogenases (ADHs).^[10–13] These two classes of enzymes require cofactor NAD(P)H regeneration which makes these strategies less suitable for commercial applications. Oxidases are an attractive class of enzymes for the production of bulk chemicals since they use oxygen either as oxidant without the need to regenerate cofactors.^[14] Alcohol oxidases are a subclass of oxidative enzymes containing either a copper or flavin adenine dinucleotide (FAD) as prosthetic group that often have a broad substrate spectrum including primary and secondary alcohols, aldehydes and ketones.^[15] A remarkable example of the power of flavoprotein oxidases is the HMF oxidase discovered

and engineered by our group. This FAD-containing biocatalyst can oxidize 5-hydroxymethylfurfural to 2,5-furandicarboxylic acid in three consecutive oxidations.^[16,17] Another recent demonstration of the biocatalytic potential of a flavoprotein alcohol oxidase was recently reported by Turner and co-workers. They showed that choline oxidase can be engineered towards a wider substrate acceptance, for the selective oxidation of primary alcohols to aldehydes.^[18]

The FAD-containing alcohol oxidase from the white-rot basidiomycete *Phanerochaete chrysosporium* (PcAOX) was recently characterized and engineered by our group for improved activity towards glycerol.^[19] The rational engineering study resulted in the variant F101S optimized for glycerol conversion. The aim of this work was to explore and expand the potential of this improved variant. In this paper, the attention was directed to diols which represent an industrially important class of chemical compounds since they are relatively cheap and can be transformed to lactones or hydroxy acids, which in turn are building blocks for biodegradable polymers. Except for a focus on diols as substrates, we also tested other alcohols (primary and secondary) and aminoalcohols. Such a large variety of different substrates was explored to probe the substrate acceptance profile of F101S-PcAOX but also to understand how it can catalyze the double oxidation of alcohols into carboxylic acids. The most striking discovery is the ability of F101S-PcAOX to selectively perform double oxidations on a subset of diols on one of the two hydroxyl groups.

The initial experiments were performed with a small set of diols, aimed to verify which variant of PcAOX (wild type PcAOX or the F101S variant) was more active (see Supporting information). NMR analysis proved to be the best method for analysis of this type of compounds. This approach is fast and it allowed a quantitative and qualitative analysis of the formed products. Table 1 presents the list of substrates tested and the product yields as determined by ¹H NMR. The results revealed that the F101S mutant outperformed the wild-type enzyme which is in line with its wider active site. F101S-PcAOX (referred to as AOX^{*} in the rest of the manuscript) was selected for further investigation.

AOX^{*} showed activity towards the shortest tested diol (1,3-propanediol), but a mixture of products was obtained. The enzyme is likely to convert this substrate to very active aldehyde and/or dialdehyde species. Substrates **1** (1,4-butanediol) was oxidized by AOX^{*} into a mixture of two products: the corresponding γ -butyrolactol and γ -butyrolactone. The lactol product intermediate was the main component and existed in buffer environment as mixture of hemiacetal and hydroxyl-aldehyde (ratio 10:1). The unanticipated finding that AOX^{*} was able to convert **1** into the lactone (by a double oxidation) triggered us to perform product analysis of other aliphatic diols. Substrate **2** was crucial to understand the reaction mechanism.

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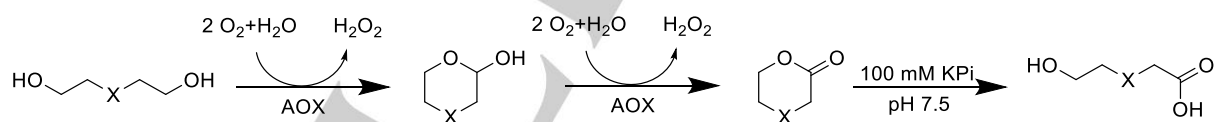
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Table 1. Results of AOX*-catalysed conversions of diols and other alcohols [a].

Entry	Substrate	Intermediate	Product	Product [%]
1				11 (63 intermediate)
2				>99[b]
3				>99
4				>99
5				>99
6				64[c] (36 aldol)
7				>99[c]
8				>99[d]
9				>99
10		/		>99
11		/		21
12		/		15
13		/	/	0

[a] Condition: substrate (20 mM), enzyme (40 μ M), 100 mM potassium phosphate buffer pH 7.5, 48 h, 35 $^{\circ}$ C. Yields were determined based on 1 H NMR. NMR was performed after the addition of D_2O (15% v/v). [b] Ratio cyclic to open product 4:1. [c] Ratio of *gem*-diol and aldehyde form of carboxylic acid (ratio ~1:1). [d] Ratio of *gem*-diol and aldehyde form of carboxylic acid (ratio ~10:1).

**Scheme 1.** Catalytic route for oxidation of 1,5-diols **3**, **4** and **5** by AOX*. X = C, S, or O.

For **2**, complete conversion was observed resulting in the doubly oxidized product. This shows that AOX* performs two consecutive oxidations of the primary alcohol group, even in the presence of a secondary alcohol moiety. The product of the double oxidation was obtained as mixture of the lactone form (γ -valerolactone) and the hydrolyzed form (4-hydroxypentanoic acid with a ratio of 4:1. Analogous to the conversion of **1**, the double oxidation of **2** presumably proceeds via formation of the corresponding lactol (5-methyltetrahydrofuran-2-ol). To verify the ability of AOX* to act on both compounds, the steady-state kinetic parameters for these and other alcohols were determined (Table 2). This indeed confirmed that AOX* can act on aliphatic diols and the lactol (5-methyltetrahydrofuran-2-ol). These data demonstrate that the catalytic route proceeds through initial lactol formation and subsequent further oxidation to the lactone.

Substrates **3**, **4** and **5** were selected as examples for 1,5-diols. For all three substrates, complete conversion was obtained and the corresponding products of the double oxidation for these diols were obtained in the form of 5-hydroxycarboxylic acids as single products. No other products (lactone, diacid or aldehyde acid) were observed by 1 H NMR. These results were in contrast to the products formed with **1** and **2**, which are obtained in the lactone form. Additional experiments were performed to explain this apparent difference. For this, we investigated the stability of the commercially available 1,4-dioxan-2-one (the lactone form of the product of substrate **4**). This compound was found to swiftly hydrolyze in buffer (Scheme 1). In buffer, in less than half hour it completely hydrolyzed to the hydroxy acid (reaction conditions: 100 mM KPi buffer pH = 7.5, see Supporting information). This indicates that, as soon as the lactone is formed upon oxidation of

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3, **4** or **5**, the formed lactone rapidly hydrolyzes into the stable hydroxy acid. We also monitored the conversion of **4**. This revealed that the first detectable product intermediate exists in its hemiacetal form. According to these data, we conclude that the AOX*-catalyzed double oxidation of aliphatic diols proceeds via formation of the corresponding hemiacetal which is subsequently oxidized into the lactone, which is prone to hydrolysis into the corresponding 5-hydroxy acids (Scheme 1).

Substrate **6** was also tested, because the corresponding product lactone (ϵ -caprolactone) is of value as polymer building block.^[20] Somewhat unforeseen, AOX* was found to oxidize both hydroxyl groups to aldehyde groups resulting in the production of adipaldehyde. In the employed buffer, adipaldehyde spontaneously reacted via aldol condensation (non-enzymatic reaction, see Supporting information) while also some further oxidation into 6-oxohexanoic acid was observed. Similarly to substrate **6**, 1,8-octanediol (**7**) and triethylene glycol (**8**) underwent oxidation on both hydroxyl groups yielding the corresponding dialdehydes, which then got further oxidized into oxocarboxylic acids. For these two substrates the aldol condensation product was not obtained. This is probably due to the unfavorable formation of a seven-membered ring product. For the latter substrates (**6-8**), AOX* is able to perform a triple oxidation by oxidizing the *gem*-diol form of the formed dialdehydes. NMR analysis also confirmed that these aliphatic aldehydes are significantly hydrated (10%-50%) in the employed buffer. For the selective oxidation on only one hydroxyl group, as observed for **3-5**, formation of a very stable hemiacetal intermediate seems to be crucial. If the hemiacetal is not formed upon the first oxidation, the enzyme will oxidize the other hydroxyl group resulting in the dialdehyde. Subsequently, one aldehyde group is oxidized to carboxylic acid via the *gem*-diol. Once that the carboxylic acid is obtained, the enzyme does not accept this compound for the further oxidation towards a diacid. Along these lines, substrate **9** was found to yield hexanoic acid, supporting our hypothesis that AOX* can further oxidize the initially formed aldehyde via its *gem*-diol. Different from the other substrates, **9** and the intermediate product formed from **9** (**Int-9**) exhibited substrate inhibition (Table 2 and Supporting information). This may be due to alternative binding pockets for these relatively apolar substrates when compared with the tested diols.

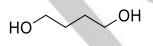
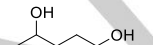
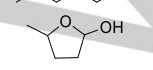
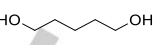
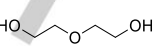
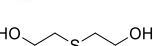
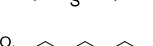
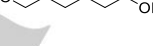
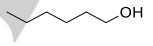
Aminoalcohol (**10**) was used to explore the substrate promiscuity of AOX*. Interestingly, complete conversion was observed for **10**. From ¹H NMR spectra it can be concluded that the reaction occurred as selective single oxidation on the hydroxyl group. A singlet at 7.88 ppm clearly indicated that the product exists as an imine (*in situ* formed spontaneously from aminoaldehyde). The ¹H NMR spectra appeared complex and the product could not be extracted with ethyl acetate to confirm the product by GC-MS analysis. Nevertheless Braekman and coworker describe that Δ -piperidine under reaction conditions (pH 7.5) analogous to ours preferably dimerizes to form tetrahydroanabasine, which correlates with our ¹H NMR spectra.^[21]

It is known that alcohol oxidases can oxidize secondary alcohols, though with poor efficiency. We selected just a few to test AOX* (substrates **11**, **12**, and **13**). Cyclohexanol (**11**) was oxidized to cyclohexanone with a low yield. Initially, we tested racemic 1-phenylethanol and its conversion was rather low, similar to cyclohexanol. After that, we tested separately each enantiomer of this alcohol (substrates **12** and **13**). Remarkably,

AOX* was highly selective towards the *S*-enantiomer of 1-phenylethanol (no conversion for the *R*-enantiomer).

The steady-state kinetic parameters for a selection of the discovered AOX* substrates were determined (Table 2). The observed values for **2** and its corresponding lactol (**int-2**) support the proposed catalytic mechanism of the double oxidation going through the lactol intermediate. The rate limiting step (lower k_{cat}/K_M) seems to be the second oxidation step, the oxidation of the lactol. This was confirmed by substrate **1** and also by shorter conversion experiments for substrates **2-5**. Shorter incubations revealed the accumulation of the respective lactols.

Table 2. Apparent Steady-State Kinetic Parameters for AOX* ^[a].

Entry	Substrate	K_M ^[a]	K_I ^[b]	k_{cat} ^[a]	k_{cat}/K_M
1		198	n.d.	6.74	34.1
2		52.6	n.d.	1.28	24.3
int-2		95.5	n.d.	1.13	11.8
3		69.6	n.d.	3.48	50.0
4		12.3	n.d.	0.73	59.3
5		119	n.d.	3.56	29.9
6		12.9	n.d.	4.07	316
9		3.0	56	0.56	187
Int-9		0.20	5.5	0.15	750

[a] Values obtained using the HRP-coupled assay in 50 mM potassium phosphate, pH 7.5. K_M values are presented in units of mM, k_{cat} values are presented in units of s⁻¹, and k_{cat}/K_M values are presented in units of M⁻¹ s⁻¹.

[b] When no substrate inhibition was observed, this is indicated as n.d. (not detected).

Substrates **4** and **5** were also tested as substrates with several other flavoprotein alcohol oxidases: alditol oxidase (HotAldO) from *Acidothermus cellulolyticus* 11B, chito oligosaccharide oxidase (ChitO) from *Fusarium graminearum*, 5-hydroxymethylfurfural oxidase (HMFO) wild type and variant 8BxHMFO from *Methylovorus* sp. strain MP688, methanol oxidase from *Hansenula* sp. (EC 1.1.3.13), glucose oxidase from *Aspergillus niger* (EC 1.1.3.4), and choline oxidase wild type and an engineered choline oxidase variant (AcCO6) from *Arthrobacter chlorophenolicus*.^[16,17,22-24] Except for (AcCO6), all these oxidases did not convert **4** and **5**. The six fold mutant of choline oxidase, AcCO6, proved to be able to perform the double oxidation of **4** and **5**, albeit with low yields: 5% product (38% lactol intermediate) with **4**, and 28% of product (68% lactol intermediate) with **5**, using the same reaction conditions as with AOX*. Furthermore, AcCO6 was found to suffer from severe substrate inhibition.

In conclusion, this work demonstrates the potential of AOX* as biocatalyst to produce, in one pot, hydroxy acids from 1,5-diols through a selective double oxidation. By this, diethylene glycol and thiodiglycol can be converted into the corresponding hydroxy

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acids, which represent interesting building blocks for biodegradable polymers. Similarly, 1,4-diols are converted into the respective lactones. The final oxidation products obtained, γ -butyrolactone and γ -valerolactone are used in the polymer industry.^{[4] [25]} The catalytic mechanism of AOX* with these diols involves the *in situ* formation of stable hemiacetals. Longer diols can also be oxidized by AOX*, resulting in the corresponding oxocarboxylic acids through a triple oxidation. For all these AOX*-catalyzed oxidation cascade reactions, no external cofactor is required. These results suggest that AOX* holds a great promise as a biocatalyst for selective oxidations.

Experimental Section

AOX* was expressed in *E. coli* NEB 10 β as His-tag-SUMO phusion using a pBAD expression vector and purified using affinity chromatography as described before.^[19] Kinetic parameters were determined using a HRP-coupled assay as described before.^[19] ¹H NMR spectra were recorded on an Agilent 400-MR spectrometer (¹H and ¹³C resonances at 400 MHz and 100 MHz, respectively). Chemical shifts are reported in parts per million (ppm) and coupling constants (J) are reported in hertz (Hz).

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Keywords: biocatalysis • diols • hydroxy acids • lactones • selective oxidation

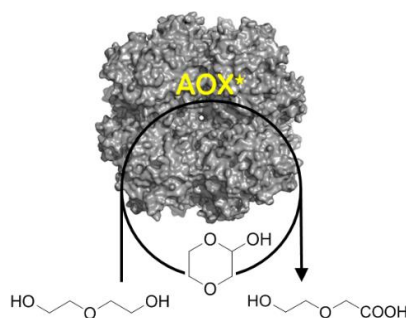
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Entry for the Table of Contents

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A flavoprotein alcohol oxidase (AOX*) can be used to perform double and triple oxidations of diols.



Caterina Martin⁺, Milos Trajkovic⁺ and Marco W. Fraaije^{*}

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